

WO9429459

Publication Title:

STRESS PROTEINS AND USES THEREFOR

Abstract:

The present invention relates to stress proteins and methods of modulating an individual's immune response. In particular, it relates to the use of such stress proteins in immune therapy and prophylaxis, which results in an induction or enhancement of an individual's immune response and as an immunotherapeutic agent which results in a decrease of an individual's immune response to his or her own cells. The present invention also relates to compositions comprising a stress protein joined to another component, such as a fusion protein in which a stress protein is fused to an antigen.

Data supplied from the esp@cenet database - <http://ep.espacenet.com>

This Patent PDF Generated by Patent Fetcher(TM), a service of Stroke of Color, Inc.

ATTORNEY DOCKET NUMBER: 8449-429-999
SERIAL NUMBER: 10/053,520
REFERENCE: **B05**

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/62, C07K 15/04, A61K 39/295, 39/04	A1	(11) International Publication Number: WO 94/29459 (43) International Publication Date: 22 December 1994 (22.12.94)
(21) International Application Number: PCT/US94/06362 (22) International Filing Date: 6 June 1994 (06.06.94) (30) Priority Data: 08/073,381 4 June 1993 (04.06.93) US (71) Applicant: WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH [US/US]; Nine Cambridge Center, Cambridge, MA 02142 (US). (72) Inventor: YOUNG, Richard, A.; 5 Sawmill Brook Road, Winchester, MA 01890 (US). (74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).	(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>	
(54) Title: STRESS PROTEINS AND USES THEREFOR (57) Abstract The present invention relates to stress proteins and methods of modulating an individual's immune response. In particular, it relates to the use of such stress proteins in immune therapy and prophylaxis, which results in an induction or enhancement of an individual's immune response and as an immunotherapeutic agent which results in a decrease of an individual's immune response to his or her own cells. The present invention also relates to compositions comprising a stress protein joined to another component, such as a fusion protein in which a stress protein is fused to an antigen.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

-1-

STRESS PROTEINS AND USES THEREFORDescriptionBackground of the Invention

Although the function of stress proteins is not
5 entirely clear, it appears that some participate in
assembly and structural stabilization of certain cellular
and viral proteins, and their presence at high
concentrations may have an additional stabilizing effect
during exposure to adverse conditions. Neidhardt, F.C.
10 and R.A. Van Bogelen, In: Escherichia coli and Salmonella
typhimurium, Cellular and Molecular Biology, (eds.
Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B.
Schaechter, M. and Umbarger, H.E. (Am. Soc. Microbiol.,
Washington, D.C.), pp. 1334-1345 (1987); Pelham, H.R.B.
15 Cell, 46:959-961 (1986); Takano, T. and T. Kakefuda,
Nature, 239:34-37 (1972); Georgopoulos, C. et al., New
Biology, 239:38-41 (1972). Phagocytic host cells produce
a hostile environment of foreign organisms, and the
ability to produce stress proteins has been implicated in
20 the survival of bacterial pathogens within macrophages
Christman, M.F. et al., Cell, 41:753-762 (1985).

Mycobacterium (M.) tuberculosis and Mycobacterium
(M.) leprae are the etiologic agents of tuberculosis and
leprosy, respectively. These diseases afflict 20-30
25 million people and continue to present a significant
global health problem. Joint International Union Against
Tuberculosis and World Health Organization Study Group,
Tubercle, 63:157-169 (1982); Bloom, B. and T. Godal, Rev.
Infect Dis. 5:765-780 (1983). To develop more effective
30 tools for the diagnosis and prevention of these diseases,
it is important to understand the immune response to
infection by mycobacterial pathogens.

-2-

The antibody and T-cell responses to infection or inoculation with killed mycobacteria have been studied in humans and in animals. Human patients with tuberculosis or leprosy produce serum antibodies directed against at least 12 mycobacterial proteins. Some of these proteins are also recognized by well-characterized murine monoclonal antibodies. Mice immunized with mycobacterial lysates produce antibodies that are directed predominantly to six M. tuberculosis and six M. leprae protein antigens.

10 Engers, H.D. Infect. Immun., 48:603-605 (1985); Engers, H.D., Infect. Immun., 51:718-720 (1986). Genes encoding these 12 mycobacterial antigens have been cloned, and recombinant proteins produced from these clones have been used to investigate the human T-lymphocyte response to

15 mycobacterial infection. Husson, R.N. and R.A. Young, Proc. Natl. Acad. Sci., USA, 84:1679-1683 (1987); Young, R.A. et al., Nature, 316:450-452 (1985); Britton, W.J. et al., Lepr. Rev., 57, Suppl. 2, 67-75 (1986).

Protection against mycobacterial disease involves

20 cell-mediated immunity. Joint International Union Against Tuberculosis and World Health Organization Study Group, Tubercle, 63:157-169 (1982); Hahn, H. and S.H.E. Kaufman, Rev. Infect. Dis., 3:1221-1250 (1981). T-lymphocytes cloned from patients or from volunteers immunized with

25 killed mycobacteria have been tested for their ability to recognize the recombinant mycobacterial proteins. Lymphocyte-proliferation assays demonstrate that most of the antigens identified with monoclonal antibodies are involved in the T-cell response to mycobacterial infection

30 or vaccination in mice and in humans. Limiting dilution analysis indicates that 20% of the mycobacterial-reactive CD4⁺ T-lymphocytes in mice immunized with M. tuberculosis recognize a single protein, the 65-kDa antigen. Kaufman, S.H.E. et al., Eur J. Immunol., 17:351-357 (1987).

-3-

Summary of the Invention

The present invention relates to stress proteins and methods of modulating an individual's (such as a human, other mammal or other vertebrate) immune response. In particular, it relates to the use of such stress proteins in immune therapy or prophylaxis, which results in an induction or enhancement of an individual's immune response and as an immunotherapeutic agent which results in a decrease of an individual's response to his or her own cells. In the embodiment in which an individual's immune response is induced or enhanced, the induced or enhanced response can be a response to antigens, such as those derived from a pathogen or cancer cell, or can be upregulation of the individual's immune status, such as in an immune compromised individual. In immune prophylaxis, stress proteins are administered to prevent or reduce the effects in an individual of a pathogen, which can be any virus, microorganism, parasite or other organism or substance (e.g., a toxin or toxoid) which causes disease or to prevent or reduce the effects in an individual of cancer cells. In preventing or reducing adverse effects of pathogens which contain stress proteins (e.g., bacteria, parasite, fungus) according to the method of the present invention, an individual's immune response to the pathogen's stress protein(s) is induced or enhanced through the administration of a vaccine which includes the pathogen's stress protein(s) or other stress proteins. The stress protein can be administered alone, as a member or component of a conjugate (e.g., joined to another antigen by chemical or recombinant means such as joined to a fusion partner resulting in a fusion protein), or as an adjuvant or carrier molecule to enhance or obtain a desired immune response to an antigen. The present invention also relates to compositions comprising a stress protein joined to another component, such as a fusion

-4-

protein in which a stress protein is fused to an antigen. Preventing or reducing adverse effects of viral pathogens which do or do not contain stress proteins, as well as preventing or reducing the adverse effects of cancer cells according to the present method, is effected by enhancing an individual's immune surveillance system. Enhancement of immune response can be effected by modulating the immune cells by stimulation with a stress protein (e.g., a bacterial stress protein).

10 In the embodiment in which an individual's immune response is decreased, such as is used in treating autoimmune diseases, stress proteins known to be involved in the autoimmune response are administered to turn down an individual's immune response by tolerizing the individual to the stress proteins. Alternatively, the immune response to stress protein, which is known to occur in autoimmune disease, is reduced by interfering with the ability of immune cells which respond to stress proteins to do so.

20 A selected stress protein of the present invention can be administered to an individual, according to the method of the present invention, and result in an immune response which provides protection against subsequent infection by a pathogen (e.g., bacteria, other infectious agents which produce stress proteins) or reduction or prevention of adverse effects of cancer cells. Alternatively, a selected stress protein can be administered to an individual, generally over time, to induce immune tolerance against the selected stress protein. For example, a selected stress protein can be administered in multiple doses over time in order to induce immune tolerance against an autoimmune disease such as rheumatoid arthritis.

-5-

Brief Description of the Drawings

Figure 1 is a graphic representation of the homologies between mycobacterial antigens and known stress proteins. Figure 1A is a representation of sequence similarity between portions of the M. tuberculosis 71-kDa antigen (residues 1-204; TB 71 kDa) and the E. coli DnaK protein (residues 430-639). Figure 1B is a representation of sequence similarity between portions of the M. tuberculosis 65-kDa antigen (residues 1-540; TB 65 kDa) and the E. coli GroEL protein (residues 1-547).

Figure 2 is a comparison of the amino acid sequence of the human P1 protein (573 residues) (SEQ ID NO: 1) and the amino acid sequence of the groEL protein (547 residues) (SEQ ID NO: 2).

Figure 3 is a comparison of the amino acid sequence of the human P1 protein (573 residues) (SEQ ID NO: 1), which is a homolog of groEL protein, and the amino acid sequence of the 65 kDa M. leprae protein (540 residues) (SEQ ID NO: 3).

Figure 4 is a comparison of the amino acid sequence of the human P1 protein (573 residues) (SEQ ID NO: 1), which is a homolog of the groEL protein, and the amino acid sequence of the 65kDa M. tuberculosis protein (540 residues) (SEQ ID NO: 4).

Figure 5 is a schematic representation of selected stress protein fusion vectors which contain a polylinker with multiple cloning sites permitting incorporation of a gene of interest.

Figure 6 is a schematic representation of the stress protein fusion vector, pKS70 containing the T7 RNA polymerase promoter, a polylinker and the *mycobacterium tuberculosis* hsp70 gene, and the stress protein fusion vector pKS72 containing the HIV p24 gag gene subcloned into the pKS70 vector.

-6-

Figure 7 is a graph illustrating the anti-p24 antibody titer in mice injected with the p24-hsp70 fusion protein, p24 alone and hsp70 alone.

Detailed Description of the Invention

- 5 Cells respond to a variety of stressful stimuli by increasing the synthesis of specific stress proteins. The most extensively studied cellular response to stressful stimuli is the synthesis of heat shock proteins (hsp) by a cell, induced by a sudden increase in temperature.
- 10 Because many of the heat shock proteins are also induced by other stresses, they are frequently called stress proteins. Stress proteins and their relatives appear to help assemble and disassemble protein complexes. In bacteria, the major stress proteins, hsp70 and hsp60,
- 15 occur at moderate levels in cells that have not been stressed but accumulate to very high levels in stressed cells. For example, hsp70 and hsp60 normally account for 1-3% of total E. coli protein, but can accumulate to about 25% under stressful conditions. Eukaryotic hsp70 and
- 20 hsp60 proteins do not accumulate to these extreme levels. Their levels range from undetectable to moderately abundant, depending on the organism and cell type.

- The present invention is based on the observation that stress proteins are among the major antigens
- 25 available for presentation to T lymphocytes and may be common immune targets in a broad spectrum of infectious diseases. Immune responses to stress proteins are involved in immune surveillance by the body and a variety of different T cell types has been shown to recognize
- 30 highly conserved stress protein determinants. Several observations, described below, suggest a model of immune surveillance in which self-reactive T cells provide a first line of defense against infection or other invasion by pathogens, which include, but are not limited to,

-7-

viruses, microorganisms, other organisms, substances such as toxins and toxoids, and agents which cause cell transformation, by recognizing and helping to eliminate stressed autologous cells, as well as cells infected with intracellular pathogens. Without wishing to be bound by this model, it is presented as one means by which it is possible to explain why prokaryotic and eukaryotic cells respond to a variety of potentially damaging stimuli, such as elevated temperature, by increasing the synthesis of a family of proteins, referred to as stress proteins, which are among the most highly conserved and abundant proteins found in nature.

Investigation of antigens involved in the immune response to the tuberculosis and leprosy bacilli (M. tuberculosis and M. leprae) initially led to the observation that a variety of stress proteins are among the major targets of the immune response, as is described at greater length below.

Further assessment has demonstrated that stress proteins may be common immune targets in a broad spectrum of infectious diseases. Sequence analysis has revealed 70-kDa heat shock protein homologues among major antigens of the protozoan parasites Plasmodium falciparum (Bianco, A.E. et al., Proc. Natl. Acad. Sci., USA, 83:8713-8717 (1986)) and Schistosoma mansoni (Hedstrom, R. et al., J. Exp. Med., 165:1430-1435 (1987)) and the malarial parasite Brugia malayi (Selkirk, M.E. et al., J. Cell Biochem., 12D:290 (1988)). Similarly, homologues of GroEL have been found among antigens involved in the immune response to Salmonella typhimurium and Coxiella (Vodkin, M.H. and J.C. Williams, J. Bacteriol., 170:1227 (1988)), as well as Bordetella pertussis (Del Giudice, G., et al., J. of Imm., 150: 2025-2032 (1993)). The presence of stress proteins among major immune targets in a variety of human pathogens is support for the idea that the stress response may be a

-8-

general component of infection and that stress proteins should be considered among candidates for subunit vaccines. All organisms respond to heat by inducing synthesis of heat shock proteins (hsp), which are a group of proteins. This response is the most highly conserved genetic system known and has been shown to occur in every organism, including microorganisms, plants and animals, investigated to date. Many of the characteristics of the response are common to all organisms and the hsp are among the most highly conserved proteins known. For example, hsp90 family and hsp70 family proteins are present in widely diverse organisms. The proteins in each family--even in such diverse organisms--show approximately 50% identity at the amino acid level and at the nonidentical residues, exhibit many similarities. Several of the proteins induced by heat are also induced by a variety of other stresses. The hsps or a closely related/similar protein are present in all organisms at normal temperatures and have been shown to have key functions in normal cell metabolism. Lindquist, S. and E.A. Craig, Ann. Rev. Genet., 22:631-677 (1988). Because the stress response is common to prokaryotes and eukaryotes and stress proteins are among the most highly conserved in sequence, it is reasonable to expect that an antigen from one pathogen could immunize against another pathogen. Exposure to foreign stress proteins early in life might, in fact, induce a degree a immunity to a variety of infectious agents. If so, this could provide an explanation for the observation that, for many pathogens, only a fraction of infected individuals actually acquire clinical disease.

The following is a description of the relationship which has been observed between stress proteins and the immune response to mycobacterial infection; of the observation and supporting information that stress

-9-

proteins are immune targets in many infections by pathogens; of the role of stress proteins as immune targets in transformed cells; of recognition of the fact that the immune response to conserved stress protein determinants may play an important role in autoimmune pathology in rheumatoid arthritis, as well as in adjuvant arthritis; and of the role of stress proteins in immune surveillance, as well as a model proposed for immune surveillance in which self-reactive T cells provide a first line of defense against infection and cell transformation.

Mycobacterial Stress Proteins are Targets of the Immune Response

An intriguing relationship between stress proteins and the immune response to mycobacterial infection has been observed. A more detailed examination of stress protein determinants and immune response mechanisms is essential to understanding the relationship among stress proteins, infection, and immunity.

In view of the involvement of proteins of M. tuberculosis and M. leprae in humoral and cell-mediated immune responses and to establish the functions of these proteins in the mycobacterial cell, the DNA encoding several of the M. tuberculosis and M. leprae antigens have been sequenced. The results, discussed in Example 1, demonstrate that many of these mycobacterial protein antigens exhibit striking sequence similarity to known stress-induced proteins. Three of the M. leprae and two of the M. tuberculosis protein antigens studied have been shown to exhibit striking sequence similarity to known stress proteins. For reasons discussed in Example 1, it is concluded that two of the M. leprae and two of the M. tuberculosis antigens are homologues of the E. coli DnaK and GroEL proteins.

-10-

In mice, immunization with mycobacterial lysates elicits antibody responses to at least six M. tuberculosis protein antigens and a similar number of M. leprae protein antigens. Monoclonal antibodies specific for these proteins have been used to isolate clones from λ gt11 DNA expression libraries of M. tuberculosis and M. leprae. The sequence of the DNA clones revealed that mycobacterial hsp70 (alias 70 kDa antigen) and hsp60 (alias 65 kDa antigen, GroEL) were the major targets of the murine antibody response to both M. tuberculosis and M. leprae. Two additional hsp, an 18 kDa member of the small hsp family and a 12 kDa homologue of groES, were found among the M. leprae and M. tuberculosis antigens. Young, D.B., et al., Proc. Natl. Acad. Sci., USA, 85:4267-4270 (1988); Shinnick, T.M., et al., Nuc. Acids Res., 17:1254 (1989).

The mycobacterial stress proteins are among the immunodominant targets of both murine antibody and T cell responses. In one study which summarized results obtained from 10 laboratories, a collection of 24 murine monoclonal antibodies recognized 6 M. leprae proteins; 7 of these antibodies are directed against 6 different determinants in the M. leprae hsp60. Engers, H.D., et al., Infect. Immun., 48:603-605 (1985); Mehra, V., et al., Proc. Natl. Acad. Sci., USA, 83:7013-7017 (1986). In a similar study, 3 of 33 monoclonal antibodies raised against M. tuberculosis recognized the M. tuberculosis hsp60 protein. Engers, H.D., et al., Infect. Immun., 51:718-720 (1986). Finally, limiting dilution analysis indicates that 20% of the mycobacterial-reactive CD4+ T lymphocytes in mice immunized with M. tuberculosis recognize this antigen. Kaufmann, S.H., et al., Eur. J. Immunol., 17:351-357 (1987).

Although a rigorous quantitative analysis of the human immune response to mycobacterial stress proteins has not yet been reported, mycobacterial stress proteins are

-11-

recognized by human antibodies and T lymphocytes and the evidence suggests that these proteins are among the major targets of the human cell mediated immune response.

- Emmrich, F., et al., J. Exp. Med., 163:1024-1029 (1985);
- 5 Mustafa, A.S., et al., Nature (London). 319:63-66 (1986);
- Oftung, F., et al., J. Immunol., 138:927-931 (1987); Lamb, J.R., et al., EMBO J., 6:1245-1249 (1987). T lymphocytes from patients with mycobacterial infection or from
- volunteers immunized with mycobacteria have been cloned
- 10 and tested for their ability to recognize the mycobacterial stress proteins. In each of these studies, some fraction of the human T cell clones were shown to recognize one or more of the mycobacterial stress proteins.

15 Stress Proteins are Immune Targets in Infections by Pathogens

- The observation that stress proteins are important targets of the immune response to mycobacterial infection and the knowledge that the major stress proteins are
- 20 conserved and abundant in other organisms suggested that stress proteins are likely to be immune targets in many infections by pathogens. Indeed, that is now clearly the case. Antigens from a wide variety of infectious agents have been identified as members of stress protein
- 25 families. The major stress protein antigen recognized by antibodies in bacterial infections is hsp60. "Common antigen", an immunodominant protein antigen long known to be shared by most bacterial species, turns out to be hsp60. Shinnick, T.M., et al., Infect. Immun., 56:446
- 30 (1988); Thole, J.E.R., et al., Microbial Pathogenesis, 4:71-83 (1988). Stress proteins have also been identified as immune targets in most major human parasite infections. Bianco, A.E., et al., Proc. Natl. Acad. Sci. USA, 83:8713 (1986); Nene, V., et al., Mol. Biochem. Parasitol., 21:179

-12-

(1986); Ardeshir, F., et al., EMBO J., 6:493 (1987); Hedstrom, R., et al., J. Exp. Med., 165:1430 (1987); Selkirk, M.E., et al., J. Cell Biochem., 12D:290 (1988), Engman, D.M., et al., J. Cell Biochem., 12D: Supplement, 5 290 (1988); Smith, D.F., et al., J. Cell Biochem., 12D:296 (1988). Antibodies to hsp70 have been identified in the sera of patients suffering from malaria, trypanosomiasis, leishmaniasis, schistosomiasis and filariasis. Hsp90 is also a target of antibodies in trypanosomiasis and a 10 member of the small hsp family is recognized in some patients with schistosomiasis.

Proteins homologous to stress proteins have also been identified in viruses. Recently, a protein encoded by the RNA genome of the Beet Yellows Closterovirus, a plant 15 virus, has been shown to be homologous to hsp70. Agranovsky, A.A., et al., J. Mol. Biol., 217: 603-610 (1991). In addition, stress protein induction occurs in eukaryotic cells following infection by diverse viruses in vitro. Collins, P.L., and Hightower, L.E., J. Virol., 20 44:703-707 (1982); Nevins, J.R., Cell, 29:913-939 (1982); Garry, R.F. et al., Virology, 129:391-332 (1988); Khandjian, E.W. and Turler, H., Mol. Cell Biol., 3:1-8 (1983); LaThangue, N.B., et al., EMBO J., 3:267-277 (1984); Jindal, S. and Young, R., J. Viral, 66:5357-5362 25 (1992). CTL that recognize these neo-antigens could limit the spread of virus by killing infected cells, possibly before substantial amounts of mature virus are assembled; and by secreting the lymphokine γ -interferon. Pestka, S., in: Methods Enzymol., Interferons, Part A., Vol. 79 30 Academic Press, New York, pp. 667 (1981). Evidence consistent with this idea is emerging. Koga et al., (1989) have shown that infection of primary murine macrophages with CMV rendered them susceptible as targets for MHC-I restricted CD8⁺ CTL specific for linear epitopes 35 of M. tuberculosis hsp60. Koga, T., et al. (1989).

-13-

Although the epitope recognized by these CTL on infected macrophages was not defined, it is tempting to speculate that a cross-reactivity with self hsp60 epitopes is being observed. Indeed, the same groups showed that a
5 homologous hsp60 is constitutively present in macrophages and is upregulated by γ -interferon stimulation.

Stress Proteins as Immune Targets in Transformed Cells

Stress proteins appear to be produced at high levels in at least some transformed cells. Bensaude, O. and
10 Morange, M., EMBO J., 2: 173-177 (1983). An 86 kDA murine tumor antigen has been found to be homologous to representatives of the hsp90 family in yeast and Drosophila. Ullrich, S.J., Proc. Natl. Acad. Sci., USA,
83: 3121-3125 (1986). Immunization of mice with the
15 purified protein led to inhibition of tumor growth in 95% of experimental animals that had been seeded with cultured tumor cells. All of the protected mice had high titers of anti-hsp90 serum antibody which was able to precipitate murine hsp90 from lysates of heat shocked mouse embryo
20 cells. Again, a role for autoreactive lymphocytes is implied, since T cells capable of recognizing autologous cells stressed by transformation could help eliminate nascent tumor cells.

Stress Proteins and Autoimmune Processes

25 Rheumatoid arthritis is characterized by a chronic proliferative and inflammatory reaction in synovial membranes which is thought to involve autoimmune processes. Rat adjuvant arthritis resembles human rheumatoid arthritis in many respects, and has been used
30 as an experimental animal model for human disease. Pearson, C.M., Arthritis Rheum., 7:80-86 (1964). Adjuvant arthritis can be induced in rats with a single intradermal injection of killed M. tuberculosis in complete Freund's

-14-

adjuvant. An autoimmune process involving T lymphocytes appears to be responsible for the generation of the disease. Holoshitz, J., et al., Science, 219:56-58 (1983). T cell lines isolated from the draining lymph nodes of arthritic rats and propagated in vitro by stimulation with M. tuberculosis-pulsed syngeneic antigen presenting cells can cause a transient form of the disease when transferred to irradiated rats. Since care was taken in these experiments to exclude the transfer of contaminating M. tuberculosis, this result strongly suggests that the clinical effects of the disease are a consequence of an autoimmune reaction in which the autoantigen is shared with M. tuberculosis.

The rat and M. tuberculosis antigens recognized by the arthritogenic T cells have been sought for a number of years. A number of different proteins present in synovial membranes have been proposed to be the cross-reactive rat antigen, but were later discounted as procedures for the purification of these proteins improved. van Eden, W., et al., Proc. Natl. Acad. Sci., USA, 82:5117-5120 (1985); Holoshitz, J., et al., Science, 219:56-58 (1983). The M. tuberculosis antigen recognized by the arthritogenic T cells was recently shown to be a 65 kDa protein (van Eden, W., et al., Nature, 331:171 (1988), which has now been shown to be hsp60 (see the Example 1). Using a combination of truncated recombinant 65 kDa proteins and peptides, a nine amino acid epitope of hsp60 has been identified as the minimum stimulatory sequence for arthritogenic T cell clones in proliferation assays. Now that it is clear that some arthritogenic T cells recognize the mycobacterial hsp60, it is quite possible that the rat autoantigen is also hsp60.

The results obtained in the adjuvant arthritis model led investigators to determine whether T lymphocytes from human rheumatoid arthritis patients also recognize

-15-

mycobacterial antigens. These investigators have found not only that patients with rheumatoid arthritis have T cells that recognize M. tuberculosis antigens, but that these T cells have diverse phenotypes. Substantial

5 proliferative responses to mycobacterial extracts are observed with uncloned T cells (predominantly CD4⁺) from both synovial infiltrates and peripheral blood, although responses are generally greater in synovial infiltrates. Abrahamson, T.G., et al., Scand. J. Immunol., 7:81-90

10 (1978); Holoshitz, J., et al., Lancet ii, 305-306 (1986). Holoshitz et al. found that 4 of 5 T cell clones isolated from human rheumatoid synovia which respond to M. tuberculosis antigens were CD4⁺ CD8⁻ cells with γ/δ T cell receptors. Holoshitz, J., et al., Nature, 339:226-229

15 (1989). This observation is interesting because γ/δ T cells have yet to be assigned a role in immunity. One of the γ/δ clones was tested for its ability to respond to purified mycobacterial hsp60 and was found to be positive in proliferation assays. Due to the conserved nature of

20 stress proteins, these T cells have the potential for autoreactivity. Lamb and coworkers have shown that polyclonal T cells from synovial infiltrates recognize both mycobacterial hsp60 and hsp70. Lamb, J.R., et al., Intl. Immunol., in press (1989). The population of T

25 cells that recognize the mycobacterial stress proteins were shown to respond to E. coli hsp60 and hsp70 and, most interestingly, human hsp70 purified from heat shocked macrophages. Thus, immune responses to conserved stress protein determinants, perhaps initiated by bacterial

30 infection (not necessarily by mycobacteria), may play an important role in autoimmune pathology in rheumatoid arthritis, as well as in adjuvant arthritis.

Stress Proteins and Immune Surveillance

-16-

Stress Proteins and Immune Surveillance

A variety of different T cell types has now been shown to recognize highly conserved stress protein determinants. The ability of cells to respond to stress by increasing the levels of the highly conserved stress proteins; the presence of T cells of diverse phenotypes in healthy individuals that are capable of recognizing self stress protein determinants; and observations that stress responses are induced by pathogenic infections and by cell transformation, all suggest a model of immune surveillance in which self-reactive T cells provide a first line of defense against infection and transformation by recognizing and helping to eliminate stressed autologous cells, as well as cells infected with intracellular pathogens. The pool of lymphocytes that recognize conserved stress protein determinants might be induced during establishment of natural microbial flora on the skin and in the gut, and maintained by frequent stimulation by pathogens, such as bacteria and viruses, as well as other stressful stimuli encountered during a normal lifetime. This model is attractive because it provides a way in which the immune system could exploit the existence of conserved epitopes in stress proteins to respond immediately to antigenically diverse pathogens and cellular changes, producing an initial defense that need not await the development of immunity to novel antigens.

The lymphocytes which recognize conserved stress protein determinants must be capable of discriminating between normal and stressed cells. Since many stress proteins are constitutively expressed in normal cells, although at lower levels than in stressed cells, the potential for autoreactivity is ever-present. Normal cells may escape destruction by expressing only substimulatory levels of stress protein determinants on their surfaces. In addition, stress proteins may only be

-17-

processed and presented during stress, and it may be relevant that many stress proteins have altered intracellular locations during stress. Finally, immune regulatory networks may prevent activation of autoreactive T cells under normal conditions. The regulatory constraints required by this system might occasionally break down, perhaps during stress caused by bacterial or viral infections, leading to autoimmune disease. Rheumatoid arthritis may be such a disease.

10 Modulation of Immune Response

The precise relationship between stress proteins and the host immune response to infection is as yet undefined. When cells are subjected to a variety of stresses, they respond by selectively increasing the synthesis of a limited set of stress proteins. Some stress proteins, including the products of DnaK and GroEL, are major constituents of the cell under normal growth conditions and are induced to even higher levels during stress. Lindquist, S., Annu. Rev. Biochem. 55: 1151-1191 (1986); Neidhardt, F.C. and R.A. VanBogelen, In Escherichia coli and Salmonella Typhimurium, Cellular and Molecular Biology, (eds. Neidhardt, F.C., Ingraham, J.L. Low, K.B. Magasanik, B. Schaechter, M. and Umbarger, H.E.) Am. Soc. Microbiol., Washington, D.C., pp. 1134-1345 (1987). It has now been demonstrated that stress-related proteins are targets of the immune response. Young, D. et al., Proc. Natl. Acad. Sci. USA, 85:4267-4270 (1988). It is reasonable to expect that immunodominant antigens would be found among such abundant proteins, as has now been shown to be the case.

According to the method of the present invention, it is possible to modulate the immune response in an individual, such as a human, other mammal or other vertebrate, by altering the individual's response to

-18-

stress proteins. In particular, it is possible to enhance or induce an individual's response to a pathogen (e.g., bacteria, virus, parasites, or other organism or agent, such as toxins, toxoids) or to cancer cells or enhance or induce an upregulation of an individual's immune status (such as in an immune compromised individual or HIV-infected individual); and to decrease an individual's autoimmune response, such as occurs in some forms of arthritis. In addition, administration of a stress protein using the method of the present invention provides protection against subsequent infection by a pathogen. As demonstrated herein, stress proteins contain regions of highly conserved amino acid sequences and have been shown to be major immunodominant antigens in bacterial and other infections. Therefore, it is reasonable to expect stress proteins can be used to elicit strong immune responses against a variety of pathogens. The stress protein administered to induce or enhance an immune response to pathogens can be the stress protein of the pathogen against which an immune response is desired or other stress protein, a portion of that protein of sufficient size to stimulate the desired immune response or a protein or amino acid sequence which is the functional equivalent of the stress protein in that it is sufficiently homologous in amino acid sequence to that of the stress protein to be capable of eliciting the desired response (an immune response substantially similar to that which occurs in response to the stress protein) in the individual to whom it is administered. The term "sufficiently homologous in amino acid sequence to that of the stress protein" means that the amino acid sequence of the protein or polypeptide will generally show at least 40% identity with the stress protein amino acid sequence; in some cases, the amino acid sequence of a functional

-19-

equivalent exhibits approximately 50% identity with the amino acid sequence of the stress protein.

Any stress-induced proteins or their functional equivalents can be used by the present invention to
5 enhance or induce an immune response in an individual (e.g. a human, other mammal or vertebrate), against an infection by a pathogen, for immunotherapy against cancer cells, for generally upregulating an individual's immune status and for use in inducing immune tolerance in an
10 individual or animal.

The stress proteins of the present invention can be administered in a variety of ways to modulate the immune response of an individual (e.g., a human, other mammal or other vertebrate). In one embodiment, the stress protein
15 is administered as a vaccine which is comprised of the stress protein or a portion of the stress protein which is of sufficient size to stimulate the desired immune response. In this embodiment, the vaccine can be a "specific vaccine" which contains a specific stress
20 protein of a particular pathogen against which an immune response is desired, such as a bacterial stress protein. In this case, since the pathogen's stress proteins are distinguishable from those of the host, it is possible to induce an immunoprophylactic response specific to the
25 pathogen's stress proteins. Blander, S.J., et al., J. Clin. Invest., 91:717-723 (1993). This can be carried out by administering a vaccine which includes all or a portion (e.g., sufficient amino acid sequence to have the desired stimulatory effect on immune response) of the pathogen's
30 stress protein or of another protein having an amino acid sequence sufficiently similar to that of the stress protein sequence to stimulate the immune response to the pathogen's stress protein. Alternatively, in the case of a pathogen which does not contain stress proteins, (e.g.
35 some viruses) or in the condition of neoplasia, stress

-20-

proteins or highly conserved stress protein determinants, such as those shown to be recognized by a variety of T cells, can be administered as a type of "general" vaccine to achieve an upregulation of the immune response.

5 Administration of such a vaccine will enhance the existing immune surveillance system. For instance, a vaccine which includes a bacterial, or other stress protein can be administered to enhance the immune system which will result in an immune response against a pathogen which does
10 not contain stress proteins. Alternatively, this type of "general" vaccine can be used to enhance an individual's immune response against cancer or to generally upregulate an individual's immune status, such as in an immune compromised individual (e.g., an individual undergoing
15 chemotherapy or an HIV-infected individual). In either case of this embodiment (specific or general vaccine), the immune response to the stress protein sequence will be increased and effects of the pathogen, disease condition or immune impairment will be reduced (decreased, prevented
20 or eliminated).

In another embodiment, stress proteins can be used to enhance immune surveillance by applying local heat or any other substances or changes in condition which induce the stress response in the individual being treated. (This
25 can also be employed in conjunction with the specific vaccine, described previously, administered to enhance an immune response to a stress protein-containing pathogen or in conjunction with the general vaccine, described above, administered to enhance the immune response against a
30 pathogen which does not contain its own stress proteins, cancer, or to upregulate the immune status of an individual). For example, it is known that increased levels of stress proteins are produced in many types of cancer cells. Therefore, enhancement of the immune
35 surveillance system, using this embodiment of the present

-21-

invention as described, can be used to facilitate destruction and/or to prevent progression or establishment of cancer cells.

The method of the present invention can also be used
5 to modify or modulate an individual's response to his or her own cells (e.g., as in autoimmune diseases). There are at least two ways in which the present invention can be used immunotherapeutically. First, stress proteins, such as heat shock proteins (e.g., hsp 70 and hsp60), are
10 known to be involved in autoimmune disease. It is, thus, possible to turn down an individual's immune response, resulting in the individual becoming more tolerant of the protein. Second, because it is known that under some circumstances, one component of the immune response in
15 certain autoimmune diseases can be to stress proteins, it is possible to selectively inhibit or interfere with the ability of immune cells which normally interact with such proteins to do so. This can be done, for example, by administering monoclonal antibodies that bind to specific
20 T cell receptors and delete or disable such cells. Alternatively, rather than knocking out immune cells, the stress response in cells can be turned down by administering a drug capable of reducing a cell's ability to undergo the stress response. For example, a drug
25 targeted to or specific for heat shock transcription factor, which is needed to stimulate heat shock genes, can be administered. The transcription factor is rendered nonfunctional or subfunctional and, as a result, cells' ability to undergo the stress response is also lessened.
30 In another embodiment of the present invention, the stress protein is administered as a vaccine which is comprised of two moieties: a stress protein and another substance (referred to as an antigen, e.g. protein, peptide, carbohydrate, lipid, organic molecule) against
35 which an immune response is desired. The two moieties are

-22-

conjugated or joined to form a single unit. Conjugation can be achieved by chemical means (e.g. through a covalent bond between the stress protein and the second moiety) or, as demonstrated in Example 2, by recombinant techniques.

- 5 If recombinant techniques are used to produce the conjugate, the result is a recombinant fusion protein which includes the stress protein and the antigen in a single molecule. This makes it possible to produce and purify a single recombinant molecule in the vaccine
- 10 production process. In this embodiment, the stress protein can be seen to act as an adjuvant-free carrier, and it stimulates strong humoral and T cell responses to the substance to which the stress protein is fused. The stress protein can be conjugated to any substance against
- 15 which an immune response is desired or to a portion of the substance sufficient to induce an immune response in an individual to whom it is administered. The substance includes but is not limited to proteins, peptides, oligosaccharides, lipids, carbohydrates, organic molecules
- 20 or a combination thereof. Barrios, C. et al., Eur. J. Immun., 22:1365-1372 (1992). Recent evidence demonstrating the effectiveness of such a vaccine indicates that mycobacterial hsp70 proteins when conjugated to other proteins act as adjuvant-free
- 25 carriers. Lussow, A.R., et al., Eur. J. Immun., 21:2297-2302 (1991). The humoral immune response to some peptides conjugated to mycobacterial hsp70 administered without any adjuvant was very similar to the antibody response to the same peptides administered in Freund's complete adjuvant.
- 30 Lussow, A.R., et al., Eur. J. Immun., 21:2297-2302 (1991). Barrios, C. et al., Eur. J. Immun., 22:1365-1372 (1992). The present invention also relates to compositions comprising a stress protein joined to another component, such as a fusion protein in which a stress protein is
- 35 fused to an antigen.

-23-

As demonstrated in Example 3, the HIV p24 gag gene was subcloned into the stress protein fusion vector pKS70 (Figure 6), containing the T7 RNA polymerase promoter, a polylinker and the *mycobacterium tuberculosis* hsp70 gene.

5 The resulting vector pKS72 (Figure 6) was used to produce the p24-hsp70 fusion protein in *E. coli*. Adjuvant-free, purified p24-hsp70 fusion protein was injected into Balb/c mice and as shown in Figure 7, the anti-p24 antibody titer was 2.7 orders of magnitude higher in mice injected with

10 the p24-hsp70 fusion protein than in mice injected with p24 alone or hsp70 alone. Mice injected with p24 and the adjuvant, alum, also produced an antibody response to p24. Finally, a demonstrable T cell response was seen in mice injected with the p24-hsp70 fusion protein and in mice

15 injected with p24 alone.

In another embodiment of the present invention, the stress protein or a portion of the stress protein which is of sufficient size to stimulate an immune response or an equivalent, is administered as an adjuvant, with another

20 substance (referred to as an antigen) against which an immune response is desired. The stress protein can be used as an adjuvant with any substance or antigen against which an immune response is desired or to a portion of the substance sufficient to induce an immune response in an

25 individual to whom it is administered. The substance includes proteins, peptides, oligosaccharides, lipids, carbohydrates, organic molecules or a combination thereof.

The stress protein, stress protein portion, stress protein functional equivalent and the substance to which

30 the stress protein is fused or conjugated present in the vaccine can be produced or obtained using known techniques. For example, the stress protein or stress protein portion can be obtained (isolated) from a source in which it occurs in nature, can be produced by cloning

35 and expressing a gene encoding the desired stress protein

-24-

or stress protein portion or can be synthesized chemically or mechanically.

An effective dosage of the stress proteins of the present invention as vaccines or adjuvants, to elicit
5 specific cellular and humoral immunity to stress proteins, or to substances conjugated to the stress proteins, such as proteins or oligosaccharides, is in the range of 0.1 to 1000 ug hsp per injection, depending on the individual to whom the stress protein is being administered. Lussow,
10 A.R., et al., Eur. J. Immun., 21:2297-2302 (1991). Barrios, C. et al., Eur. J. Immun., 22:1365-1372 (1992).
The appropriate dosage of the stress protein for each individual will be determined by taking into consideration, for example, the particular stress protein
15 being administered, the type of individual to whom the stress protein is being administered, the age and size of the individual, the condition being treated or prevented and the severity of the condition. Those skilled in the art will be able to determine using no more than routine
20 experimentation, the appropriate dosage to administer to an individual.

Various delivery systems can be used to administer an effective dose of the vaccine of the present invention. Methods of introduction include, for example, intradermal,
25 intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural and oral routes. Any other convenient route of administration can be used (infusion of a bolus injection, infusion of multiple injections over time, absorption through epithelial or mucocutaneous
30 linings such as, oral mucosa, rectal and intestinal mucosa) or a series of injections over time.

The present invention is further illustrated by the following exemplification, which is not intended to be limiting in any way.

-25-

EXEMPLIFICATIONEXAMPLE 1 Isolation and Characterization of Mycobacterial Stress Protein Antigens

Recombinant DNA Clones. The isolation and
5 characterization of M. tuberculosis and M. leprae λ gt11
genomic DNA clones with murine monoclonal antibodies have
been described. Husson, R.N. and Young, R.A., Proc. Natl.
Acad. Sci., USA 84: 1679-1683 (1987); Young, R.A., et al.,
Nature (London) 316: 450-452 (1985). DNA was isolated
10 from these clones and was manipulated by standard
procedures. Davis, R.W., Advanced Bacterial Genetics: A
Manual for Genetic Engineering (Cold Spring Harbor Lab.,
Cold Spring Harbor, NY), (1980).

DNA Sequence Analysis. DNA was subcloned into vector
15 M13mp18 or M13mp19 (New England Biolabs), as suggested by
the supplier. Dideoxynucleotide chain-termination
reactions and gel electrophoresis of the sequenced
produced were as described. Davis, R.W., Advanced
Bacterial Genetics: A Manual for Genetic Engineering (Cold
20 Spring Harbor Lab., Cold Spring Harbor, NY), (1980). DNA
sequences were determined for both strands of DNA.
Computer analysis of sequences with UWGCG programs was as
described by Devereux, J., et al., Nucleic Acids Res., 12:
387-395 (1984).

25 Immunoblot Analysis. Escherichia coli strain TG1 was
transformed with the following plasmids by standard
procedures (Maniatis, T., et al., Molecular Cloning, A
Laboratory Manual (Cold Spring Harbor Lab., Cold Spring
Harbor, NY) (1982), with selection for ampicillin
30 resistance: pND5, a derivative of pBR325 containing the E.
coli GroEL genes (Jenkins, A.J., et al., Mol. Gen. Genet.,
202: 446-454 (1986); pUC8 (Vic, J., Gene, 19: 259-268
(1982); pUC8 with insert DNA for λ gt11 clone Y3178 (M.

-26-

leprae 65-kDa antigen, Young, R.A., et al., Nature, (London) 316: 450-452 (1985)) ligated in the EcoRI site.

Overnight cultures of E. coli strains in Luria-Bertani (LB) medium were centrifuged and resuspended in isotonic phosphate-buffered saline at a cell density corresponding to an absorbance of 2 at 600 nm. An equal volume of sample buffer containing 2% (wt/vol) NaDodSO₄ was added, and, after heating on a boiling water bath for 2 min, samples were electrophoresed on 12% (wt/vol) polyacrylamide gels in the presence of NaDodSO₄. Blots were prepared by electrophoretic transfer of the proteins to a nitrocellulose membrane, and binding of monoclonal antibodies was assayed with a peroxidase-conjugated secondary antibody as described. Young, D.B., et al., Infect. Immun., 55: 1421-1425 (1987).

Six M. tuberculosis and six M. leprae proteins have been implicated in the immune response to the mycobacterial pathogens (Table 1). To obtain clues to the normal cellular function of several of these mycobacterial antigens, DNA clones encoding these proteins, isolated by using monoclonal antibodies to probe lambda gt11 libraries (Husson, R.N. and Young, R.A., Proc. Natl. Acad. Sci. USA, 84: 1679-1683 (1987); Young, R.A., et al., Nature, (London) 316: 450-452 (1985)) were subjected to sequence analysis. The sequences elucidated have been submitted to the GenBank sequence database.

The Mycobacterial 71-k Da Antigen. The 71-k Da antigen of M. tuberculosis is recognized by human T cells during infection (Table 1).

-27-

TABLE 1MYCOBACTERIAL PROTEIN ANTIGENS

Protein, kDA	Recognized by Human T Cells	Subjected to sequence analysis	Homology with known proteins
<i>M. tuberculosis</i>			
71	+	+	DnaK
65*	+	+	GroEL
38	+	-	-
19	+	+	None
14	+	-	-
12	ND	-	-
<i>M. leprae</i>			
70	ND	-	DnaK
65	+	+	GroEL
36	+	-	-
28	+	-	-
18	+	+	Plant Hsp
12	ND	-	-

Mycobacterial protein antigens, their recognition by human T cells, and homology of the deduced mycobacterial protein sequences to known proteins are summarized. ND, not determined; +, yes; -, no

- 5 * Includes data derived from study of the 65-kDA antigens of *M. bovis* BCG (Bacillus Calmette-Guérin), which is identical to the *M. tuberculosis* 65-kDA antigen.
- + A.S. Mustafa, J.R. Lamb, D. Young and R.A. Young, unpublished data.

-28-

The insert DNA of lambdagt11 clone Y3271 (Husson, R.N., et al., Proc. Natl. Acad. Sci. USA, 84: 1679-1683 (1987)), was sequenced to obtain amino acid sequence information for the 71-kDa antigen of M. tuberculosis.

5 This clone produces a beta-galactosidase fusion protein containing the carboxyl-terminal one-third of the 71-kDa antigen exhibiting 40% amino acid sequence identity with the comparable segment of the dnaK gene product from E. coli (Bardwell, J.C., et al., Proc. Natl. Sci., USA, 81: 10 848-852 (1984)), (Fig. 1). Figure 1A shows the extent of sequence similarity between portions of the mycobacterial and the E. coli 70-k Da polypeptides. Sequences transcriptionally downstream from the mycobacterial 71-k Da gene predict a 356-amino acid protein homologous to the 15 E. coli dnaJ gene product (unpublished data), indicating that the E. coli dnaK-dnaJ operon structure is conserved in M. tuberculosis and consistent with the conclusion that the mycobacterial 71-kDa antigen is a homologue of the E. coli dnaK gene product. The product of the dnaK gene is a 20 member of the 70-kDa heat shock protein family that is highly conserved among prokaryotes and eukaryotes (Bardwell, J.C., et al., Proc. Natl. Acad. Sci., USA, 81: 848-852 (1984); Lindquist, S., Annu. Rev. Biochem., 55: 1151-1191 (1986)).

25 The M. leprae 70-k Da antigen cross-reacts with monoclonal antibodies directed to the M. tuberculosis 70-kDa antigen. M. tuberculosis and M. leprae are both members of the 70-k Da heat shock protein family of stress proteins.

30 The mycobacterial 65-kDa antigen. The 65-kDa antigens of M. tuberculosis and M. leprae are involved in the human T-cell response to mycobacterial infection (Table 1). Genes encoding these proteins have been isolated (Husson, R.N., and Young, R.A., Proc. Natl. Acad. Sci., USA, 84: 1679-1683 (1987); Young, R.A., et al., 35

-29-

Nature, (London) 316: 450-452 (1985)) and sequenced (Shinnick, T.M., J. Bacteriol., 169: 1080-1088 (1987); Mehram, V., et al., Proc. Natl. Acad. Sci., USA 83: 7013-7017 (1986)), revealing that the amino acid sequences of the 65-kDa antigens of M. tuberculosis (SEQ ID NO: 4) and M. leprae (SEQ ID NO: 3) are 95% identical. These proteins sequences exhibited no significant sequence similarity to proteins in the GenBank database.

Identification of these proteins was based on the observation that some monoclonal antibodies directed against the mycobacterial 65-kDa antigens cross-react with an E. coli protein of 60kDa. E. coli cells transformed with the plasmid pND5 (Sanger, F., et al., Proc. Natl. Acad. Sci., USA 74: 5463-5467 (1977), which contains the E. coli gro E genes, had been shown to accumulate large amounts of the 60-kDa protein. A comparison of the mycobacterial 65-kDa protein sequences with those determined for E. coli groEL (C. Woolford, K. Tilly, C. Georgopoulos, and R.H., unpublished data) revealed the extent of the sequence similarity as shown in Figure 1B.

The 60-kDa Gro EL protein is a major stress protein in E. coli. Lindquist, S., Annual. Rev. Biochem., 55: 1151-1191 (1986); Nature, 333: 330-334 (1988). There is some evidence that the mycobacterial 65-kDa proteins accumulate in response to stress: Mycobacterium bovis BCG (bacillus Calmette-Guerin) cultures grown in zinc-deficient medium are substantially enriched in this protein (De Bruyn, J., et al., Infect. Immun. 55: 245-252 (1987)). This infers that the 65-kDa proteins of M. tuberculosis and M. leprae are homologues of the E. coli Gro EL protein.

Other Mycobacterial Antigens. T lymphocytes that respond to the M. tuberculosis 19-kDa antigen and the M. leprae 18-kDa antigen have been observed in humans with tuberculosis and leprosy, respectively (Table 1). DNA

-30-

encoding these antigens was sequenced from the λ gt11 clones Y3148 (Husson, R.N. and Young, R.A., Proc. Natl. Acad. Sci., USA 84: 1679-1683 (1987); and Y3179 (Young, R.A., et al., Nature, (London) 316: 450-452 (1985)),
5 respectively. The M. tuberculosis 19-kDa protein sequence predicted from the DNA exhibited no significant sequence similarity to proteins in the GenBank database.

However, the M. leprae 18-kDa protein sequence was similar to the soybean 17-kDa protein heat shock protein,
10 a protein representation of a major class of plant heat shock proteins (Schoffl, F. and Van Bogelen, R.A., In: Escherichia coli and Salmonella typhimurium, Cellular and Molecular Biology, Am. Soc. Microbiol., Washington, D.C. (1987)).

15 EXAMPLE 2 Construction of Stress Protein-Fusion Vaccines for Use as Adjuvant-Free Carriers in Immunizations

Recombinant Fusion Vectors. A series of stress protein fusion vectors for use in E. coli were constructed
20 and are shown in Figure 5. These vectors contain the T7 RNA polymerase promoter fused to the M. bovis BCG hsp70 gene or the M. bovis BCG hsp60 gene. The vectors also contain a polylinker with multiple cloning sites, permitting incorporation of a gene of interest so that the
25 antigen encoded by that gene is expressed as a fusion protein with the stress protein. A subset of these vectors permit incorporation of the foreign gene with a coding sequence for a C-terminal 6-Histidine "tag" for ease of fusion protein purification. Thus far,
30 recombinant clones have been generated that produce hsp70 proteins fused to HIV gag and HIV pol proteins.

-31-

Purification of stress protein fusions. Two strategies have been developed to purify the recombinant fusion proteins. The T7 system usually produces such large amounts of protein that it forms inclusion bodies, permitting purification by centrifugation. The preliminary results indicate that an hsp70-HIV gag fusion protein accounts for about 20% of total *E. coli* protein in the T7 system. If necessary, other fusion proteins can be purified via the 6-Histidine "tag".

10 EXAMPLE 3 ADJUVANT-FREE CARRIER EFFECT OF HSP70 IN VIVO

The stress protein fusion vector pKS70 (figure 6), containing the T7 RNA polymerase promoter, a polylinker and the *mycobacterium tuberculosis* hsp70 gene, was constructed. The HIV p24 gag gene was subcloned into pKS70 using the NdeI and BamHI sites and the resulting pKS72 vector (Figure 6) was used to produce the p24-hsp70 fusion protein in *E. coli*. The fusion protein was purified as inclusion bodies and further purified using ATP-agarose chromatography and MonoQ ion exchange chromatography.

The p24-hsp70 protein in phosphate buffered saline (PBS), in the absence of an adjuvant, was injected intraperitoneally into Balb/c mice. As controls, the p24 protein alone in PBS or the hsp70 protein alone in PBS was injected into different groups of mice. Three weeks later, the mice were boosted and finally, three weeks after the boost, the mice were bled. The anti-p24 antibody titer was then determined by ELISA. Mice injected with 25 pmoles of p24-hsp70 had antibody levels 2.7 orders of magnitude higher than mice injected with p24 alone or hsp70 alone (Figure 7). Results of experiments in which mice were injected with p24 and the adjuvant, alum, also showed that there was an antibody response to p24. In addition, mice injected with the p24-hsp70 fusion

-32-

protein and mice injected with p24 alone produced a demonstrable T cell response.

Equivalents

Those skilled in the art will recognize, or be able
5 to ascertain using no more than routine experimentation,
many equivalents to the specific embodiments of the
invention described specifically herein. Such equivalents
are intended to be encompassed in the scope of the
following claims.

-33-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) Applicants: Whitehead Institute for Biomedical Research
and
Medical Research Council
- (ii) TITLE OF INVENTION: Stress Proteins and Uses Therefore
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Hamilton, Brook, Smith & Reynolds, P.C.
 - (B) STREET: 2 Militia Drive
 - (C) CITY: Lexington
 - (D) STATE: MA
 - (E) COUNTRY: USA
 - (F) ZIP: 02173
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/073,381
 - (B) FILING DATE: 04 June 1993

-34-

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Granahan, Patricia
- (B) REGISTRATION NUMBER: 32,227
- (C) REFERENCE/DOCKET NUMBER: WHI88-08AFA2

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (617) 861-6240

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 575 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met	Leu	Arg	Leu	Pro	Thr	Val	Phe	Arg	Gln	Met	Arg	Pro	Val	Ser	Arg
1				5					10					15	
Val	Leu	Ala	Pro	His	Leu	Thr	Arg	Ala	Tyr	Ala	Lys	Asp	Val	Lys	Phe
			20					25					30		
Gly	Ala	Asp	Ala	Arg	Ala	Leu	Met	Leu	Gln	Gly	Val	Asp	Leu	Leu	Ala
			35				40					45			
Asp	Ala	Val	Ala	Val	Thr	Met	Gly	Pro	Lys	Gly	Arg	Thr	Val	Ile	Ile
			50				55				60				
Glu	Gln	Ser	Trp	Gly	Ser	Pro	Lys	Val	Thr	Lys	Asp	Gly	Val	Thr	Val
65						70				75				80	

Ala	Lys	Ser	Ile	Asp	Gly	Leu	Lys	Asp	Lys	Tyr	Lys	Asn	Ile	Gly	Ala	Lys	
				85						90						95	
Leu	Val	Gln	Asp	Val	Ala	Asn	Asn	Thr	Asn	Glu	Glu	Ala	Gly	Asp	Gly		
			100					105					110				
Thr	Thr	Thr	Ala	Thr	Val	Leu	Ala	Arg	Ser	Ile	Ala	Lys	Glu	Gly	Phe		
		115					120					125					
Glu	Lys	Ile	Ser	Lys	Gly	Ala	Asn	Pro	Val	Glu	Ile	Arg	Arg	Gly	Val		
	130					135						140					
Met	Leu	Ala	Val	Asp	Ala	Val	Ile	Ala	Glu	Leu	Lys	Lys	Gln	Ser	Lys		
145					150					155					160		
Pro	Val	Thr	Thr	Pro	Glu	Glu	Ile	Ala	Gln	Val	Ala	Thr	Ile	Ser	Ala		
				165					170						175		
Asn	Gly	Asp	Lys	Glu	Ile	Gly	Asn	Ile	Ile	Ser	Asp	Ala	Met	Lys	Lys		
			180					185					190				
Val	Gly	Arg	Lys	Gly	Val	Ile	Thr	Val	Lys	Asp	Gly	Lys	Thr	Leu	Asn		
		195					200						205				
Asp	Glu	Leu	Glu	Ile	Ile	Glu	Gly	Met	Lys	Phe	Asp	Arg	Gly	Tyr	Ile		
	210					215						220					
Ser	Pro	Tyr	Phe	Ile	Asn	Thr	Ser	Lys	Gly	Gln	Lys	Cys	Glu	Phe	Gln		
225					230					235					240		
Asp	Ala	Tyr	Val	Leu	Leu	Ser	Glu	Lys	Lys	Ile	Ser	Ser	Ile	Gln	Ser		
				245					250					255			
Ile	Val	Pro	Ala	Leu	Glu	Ile	Ala	Asn	Ala	His	Arg	Lys	Pro	Leu	Val		
			260					265					270				
Ile	Ile	Ala	Glu	Asp	Val	Asp	Gly	Glu	Ala	Leu	Ser	Thr	Leu	Val	Leu		
		275					280						285				
Asn	Arg	Leu	Lys	Val	Gly	Leu	Gln	Val	Val	Ala	Val	Lys	Ala	Pro	Gly		
	290					295						300					

-36-

Phe Gly Asp Asn Arg Lys Asn Gln Leu Lys Asp Met Ala Ile Ala Thr
 305 310 315 320

Gly Gly Ala Val Phe Gly Glu Glu Gly Leu Thr Leu Asn Leu Glu Asp
 325 330 335

Val Gln Pro His Asp Leu Gly Lys Val Gly Glu Val Ile Val Thr Lys
 340 345 350

Asp Asp Ala Met Leu Leu Lys Gly Lys Gly Asp Lys Ala Gln Ile Glu
 355 360 365

Lys Arg Ile Gln Glu Ile Ile Glu Gln Leu Asp Val Thr Thr Ser Glu
 370 375 380

Tyr Glu Lys Glu Lys Leu Asn Glu Arg Leu Ala Lys Leu Ser Asp Gly
 385 390 395 400

Val Ala Val Leu Lys Val Gly Gly Thr Ser Asp Val Glu Val Asn Glu
 405 410 415

Lys Lys Asp Arg Val Thr Asp Ala Leu Asn Ala Thr Arg Ala Ala Val
 420 425 430

Glu Glu Gly Ile Val Leu Gly Gly Gly Cys Ala Leu Leu Arg Cys Ile
 435 440 445

Pro Ala Leu Asp Ser Leu Thr Pro Ala Asn Glu Asp Gln Lys Ile Gly
 450 455 460

Ile Glu Ile Ile Lys Arg Thr Leu Lys Ile Pro Ala Met Thr Ile Ala
 465 470 475 480

Lys Asn Ala Gly Val Glu Gly Ser Leu Ile Val Glu Lys Ile Met Gln
 485 490 495

Ser Ser Ser Glu Val Gly Tyr Asp Ala Met Ala Gly Asp Phe Val Asn
 500 505 510

Met Val Glu Lys Gly Ile Ile Asp Pro Thr Lys Val Val Arg Thr Ala
 515 520 525

-37-

Leu Leu Asp Ala Ala Gly Val Ala Ser Leu Leu Thr Thr Ala Glu Val
 530 535 540

Val Val Thr Glu Ile Pro Lys Glu Glu Lys Asp Pro Gly Met Gly Ala
 545 550 555 560

Met Gly Gly Met Gly Gly Xaa Xaa Gly Met Gly Gly Gly Met Phe
 565 570 575

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 575 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 1 5 10 15

Xaa Met Ala Xaa Xaa Xaa Xaa Xaa Xaa Xaa Ala Lys Asp Val Lys Phe
 20 25 30

Gly Asn Asp Ala Arg Val Lys Met Leu Arg Gly Val Asn Val Leu Ala
 35 40 45

Asp Ala Val Lys Val Thr Leu Gly Pro Lys Gly Arg Asn Val Val Leu
 50 55 60

Asp Lys Ser Phe Gly Ala Pro Thr Ile Thr Lys Asp Gly Val Ser Val
 65 70 75 80

Ala Arg Glu Ile Glu Pro Glu Asp Lys Phe Glu Asn Met Gly Ala Gln
 85 90 95

-38-

Met Val Lys Glu Val Ala Ser Lys Ala Asn Asp Ala Ala Gly Asp Gly		
100	105	110
Thr Thr Thr Ala Thr Val Leu Ala Gln Ala Ile Ile Thr Glu Gly Leu		
115	120	125
Lys Ala Val Ala Ala Gly Met Asn Pro Met Asp Leu Lys Arg Gly Ile		
130	135	140
Asp Lys Ala Val Thr Ala Ala Val Glu Glu Leu Lys Ala Leu Ser Val		
145	150	155 160
Pro Cys Ser Asp Ser Lys Ala Ile Ala Gln Val Gly Thr Ile Ser Ala		
165	170	175
Asn Ser Asp Glu Thr Val Gly Lys Leu Ile Ala Glu Ala Met Asp Lys		
180	185	190
Val Gly Lys Glu Gly Val Ile Thr Val Glu Asp Gly Thr Gly Leu Gln		
195	200	205
Asp Glu Leu Asp Val Val Glu Gly Met Gln Phe Asp Arg Gly Tyr Leu		
210	215	220
Ser Pro Tyr Phe Ile Asn Lys Pro Glu Thr Gly Ala Val Glu Leu Glu		
225	230	235 240
Ser Pro Phe Ile Leu Leu Ala Asp Lys Lys Ile Ser Asn Ile Arg Glu		
245	250	255
Met Leu Pro Val Leu Glu Ala Val Ala Lys Ala Gly Lys Pro Leu Leu		
260	265	270
Ile Ile Ala Glu Asp Val Glu Gly Glu Ala Leu Ala Thr Ala Val Val		
275	280	285
Asn Thr Ile Arg Gly Ile Val Lys Val Ala Ala Val Lys Ala Pro Gly		
290	295	300

-39-

Phe Gly Asp Arg Arg Lys Ala Met Leu Gln Asp Ile Ala Thr Leu Thr
305 310 315 320

Gly Gly Thr Val Ile Ser Glu Glu Xaa Ile Gly Met Glu Leu Glu Lys
325 330 335

Ala Thr Leu Glu Asp Leu Gly Gln Ala Lys Arg Val Val Ile Asn Lys
340 345 350

Asp Thr Thr Thr Ile Ile Asp Gly Val Gly Glu Glu Ala Ala Ile Gln
355 360 365

Gly Arg Val Ala Gln Ile Arg Gln Gln Ile Glu Glu Ala Thr Ser Asp
370 375 380

Tyr Asp Arg Glu Lys Leu Gln Glu Arg Val Ala Lys Leu Ala Gly Gly
385 390 395 400

Val Ala Val Ile Lys Val Gly Ala Ala Thr Glu Val Glu Met Lys Glu
405 410 415

Lys Lys Ala Arg Val Glu Asp Ala Leu His Ala Thr Arg Ala Ala Val
420 425 430

Glu Glu Gly Val Val Ala Gly Gly Gly Val Ala Leu Ile Arg Val Ala
435 440 445

Ser Lys Leu Ala Asp Leu Arg Gly Gln Asn Glu Asp Gln Asn Val Val
450 455 460

Ser Ser Ser Leu Xaa Arg Ala Met Glu Ala Pro Leu Arg Gln Ile Val
465 470 475 480

Leu Asn Cys Gly Glu Glu Pro Ser Val Val Ala Asn Thr Val Lys Gly
485 490 495

Gly Asp Gly Asn Tyr Gly Tyr Asn Ala Ala Thr Glu Glu Tyr Gly Asn
500 505 510

-40-

Met Ile Asp Met Gly Ile Leu Asp Pro Thr Lys Val Thr Arg Ser Ala
 515 520 525

Leu Gln Tyr Ala Ala Ser Val Ala Gly Leu Met Ile Thr Thr Glu Cys
 530 535 540

Met Val Thr Asp Leu Pro Lys Asn Asp Xaa Ala Ala Asp Leu Gly Ala
 545 550 555 560

Ala Gly Gly Met Gly Gly Met Gly Gly Met Gly Gly Met Met Xaa
 565 570 575

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 573 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 1 5 10 15

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Ala Lys Thr Ile Ala Tyr
 20 25 30

Asp Glu Glu Ala Arg Arg Gly Leu Glu Arg Gly Leu Asn Ser Leu Ala
 35 40 45

Asp Ala Val Lys Val Thr Leu Gly Pro Lys Gly Arg Asn Val Val Leu
 50 55 60

Glu Lys Lys Trp Gly Ala Pro Thr Ile Thr Asn Asp Gly Val Ser Ile
 65 70 75 80

-41-

Ala Lys Glu Ile Glu Leu Glu Asp Pro Tyr Glu Lys Ile Gly Ala Glu
85 90 95

Leu Val Lys Glu Val Ala Lys Lys Thr Asp Asp Val Ala Gly Asp Gly
100 105 110

Thr Thr Thr Ala Thr Val Leu Ala Gln Ala Leu Val Lys Glu Gly Leu
115 120 125

Arg Asn Val Ala Ala Gly Ala Asn Pro Leu Gly Leu Lys Arg Gly Ile
130 135 140

Glu Lys Ala Val Asp Lys Val Thr Glu Thr Leu Leu Lys Asp Ala Lys
145 150 155 160

Glu Val Glu Thr Lys Glu Gln Ile Ala Ala Thr Ala Ala Ile Ser Ala
165 170 175

Xaa Gly Asp Gln Ser Ile Gly Asp Leu Ile Ala Glu Ala Met Asp Lys
180 185 190

Val Gly Asn Glu Gly Val Ile Thr Val Glu Glu Ser Asn Thr Phe Gly
195 200 205

Leu Gln Leu Glu Leu Thr Glu Gly Met Arg Phe Asp Lys Gly Tyr Ile
210 215 220

Ser Gly Tyr Phe Val Thr Asp Ala Glu Arg Gln Glu Ala Val Leu Glu
225 230 235 240

Glu Pro Tyr Ile Leu Leu Val Ser Ser Lys Val Ser Thr Val Lys Asp
245 250 255

Leu Leu Pro Leu Leu Glu Lys Val Ile Gln Ala Gly Lys Ser Leu Leu
260 265 270

Ile Ile Ala Glu Asp Val Glu Gly Glu Ala Leu Ser Thr Leu Val Val
275 280 285

Asn Lys Ile Arg Gly Thr Phe Lys Ser Val Ala Val Lys Ala Pro Gly
290 295 300

-42-

Phe Gly Asp Arg Arg Lys Ala Met Leu Gln Asp Met Ala Ile Leu Thr
305 310 315 320

Gly Ala Gln Val Ile Ser Glu Glu Xaa Val Gly Leu Thr Leu Glu Asn
325 330 335

Thr Asp Leu Ser Leu Leu Gly Lys Ala Arg Lys Val Val Met Thr Lys
340 345 350

Asp Glu Thr Thr Ile Val Glu Gly Ala Gly Asp Thr Asp Ala Ile Ala
355 360 365

Gly Arg Val Ala Gln Ile Arg Thr Glu Ile Glu Asn Ser Asp Ser Asp
370 375 380

Tyr Asp Arg Glu Lys Leu Gln Glu Arg Leu Ala Lys Leu Ala Gly Gly
385 390 395 400

Val Ala Val Ile Lys Ala Gly Ala Ala Thr Glu Val Glu Leu Lys Glu
405 410 415

Arg Lys His Arg Ile Glu Asp Ala Val Arg Asn Ala Lys Ala Ala Val
420 425 430

Glu Glu Gly Ile Val Ala Gly Gly Gly Val Thr Leu Leu Gln Ala Ala
435 440 445

Pro Ala Leu Asp Lys Leu Lys Leu Thr Gly Asp Glu Ala Thr Xaa Gly
450 455 460

Ala Asn Ile Val Lys Val Ala Leu Glu Ala Pro Leu Lys Gln Ile Ala
465 470 475 480

Phe Asn Ser Gly Met Glu Pro Gly Val Val Ala Glu Lys Val Arg Asn
485 490 495

Leu Ser Val Gly His Gly Leu Asn Ala Ala Thr Gly Glu Tyr Glu Asp
500 505 510

Leu Leu Lys Ala Gly Val Ala Asp Pro Val Lys Val Thr Arg Ser Ala
515 520 525

-43-

Leu Gln Asn Ala Ala Ser Ile Ala Gly Leu Phe Thr Thr Xaa Glu Ala
 530 535 540

Val Val Ala Asp Lys Pro Glu Lys Thr Ala Ala Pro Ala Ser Asp Pro
 545 550 555 560

Thr Gly Gly Met Gly Gly Xaa Met Asp Xaa Xaa Xaa Phe
 565 570

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 573 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 1 5 10 15

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Ala Lys Thr Ile Ala Tyr
 20 25 30

Asp Glu Glu Ala Arg Arg Gly Leu Glu Arg Gly Leu Asn Ala Leu Ala
 35 40 45

Asp Ala Val Lys Val Thr Leu Gly Pro Lys Gly Arg Asn Val Val Leu
 50 55 60

Glu Lys Lys Trp Gly Ala Pro Thr Ile Thr Asn Asp Gly Val Ser Ile
 65 70 75 80

Ala Lys Glu Ile Glu Leu Glu Asp Pro Tyr Glu Lys Ile Gly Ala Glu
 85 90 95

-44-

Leu Val Lys Glu Val Ala Lys Lys Thr Asp Asp Val Ala Gly Asp Gly
 100 105 110

Thr Thr Thr Ala Thr Val Leu Ala Gln Ala Leu Arg Lys Glu Gly Leu
 115 120 125

Arg Asn Val Ala Ala Gly Ala Asn Pro Leu Gly Leu Lys Arg Gly Ile
 130 135 140

Glu Lys Ala Val Glu Lys Val Thr Glu Thr Leu Leu Lys Gly Ala Lys
 145 150 155 160

Glu Val Glu Thr Lys Glu Gln Ile Ala Ala Thr Ala Ala Ile Ser Ala
 165 170 175

Xaa Gly Asp Gln Ser Ile Gly Asp Leu Ile Ala Glu Ala Met Asp Lys
 180 185 190

Val Gly Asn Glu Gly Val Ile Thr Val Glu Glu Ser Asn Thr Phe Gly
 195 200 205

Leu Gln Leu Glu Leu Thr Glu Gly Met Arg Phe Asp Lys Gly Tyr Ile
 210 215 220

Ser Gly Tyr Phe Val Thr Asp Pro Glu Arg Gln Glu Ala Val Leu Glu
 225 230 235 240

Asp Pro Tyr Ile Leu Leu Val Ser Ser Lys Val Ser Thr Val Lys Asp
 245 250 255

Leu Leu Pro Leu Leu Glu Lys Val Ile Gly Ala Gly Lys Pro Leu Leu
 260 265 270

Ile Ile Ala Glu Asp Val Glu Gly Glu Ala Leu Ser Thr Leu Val Val
 275 280 285

Asn Lys Ile Arg Gly Thr Phe Lys Ser Val Ala Val Lys Ala Pro Gly
 290 295 300

-45-

Phe Gly Asp Arg Arg Lys Ala Met Leu Gln Asp Met Ala Ile Leu Thr
305 310 315 320

Gly Gly Gln Val Ile Ser Glu Glu Xaa Val Gly Leu Thr Leu Glu Asn
325 330 335

Ala Asp Leu Ser Leu Leu Gly Lys Ala Arg Lys Val Val Val Thr Lys
340 345 350

Asp Glu Thr Thr Ile Val Glu Gly Ala Gly Asp Thr Asp Ala Ile Ala
355 360 365

Gly Arg Val Ala Gln Ile Arg Gln Glu Ile Glu Asn Ser Asp Ser Asp
370 375 380

Tyr Asp Arg Glu Lys Leu Gln Glu Arg Leu Ala Lys Leu Ala Gly Gly
385 390 395 400

Val Ala Val Ile Lys Ala Gly Ala Ala Thr Glu Val Glu Leu Lys Glu
405 410 415

Arg Lys His Arg Ile Glu Asp Ala Val Arg Asn Ala Lys Ala Ala Val
420 425 430

Glu Glu Gly Ile Val Ala Gly Gly Gly Val Thr Leu Leu Gln Ala Ala
435 440 445

Pro Thr Leu Asp Glu Leu Lys Xaa Leu Glu Gly Asp Glu Ala Thr Gly
450 455 460

Ala Asn Ile Val Lys Val Ala Leu Glu Ala Pro Leu Lys Gln Ile Ala
465 470 475 480

Phe Asn Ser Gly Leu Glu Pro Gly Val Val Ala Glu Lys Val Arg Asn
485 490 495

Leu Pro Ala Gly His Gly Leu Asn Ala Gln Thr Gly Val Tyr Glu Asp
500 505 510

-46-

Leu Leu Ala Ala Gly Val Ala Asp Pro Val Lys Val Thr Arg Ser Ala
515 520 525

Leu Gln Asn Ala Ala Ser Ile Ala Gly Leu Phe Leu Thr Thr Glu Ala
530 535 540

Val Val Ala Asp Lys Pro Glu Lys Glu Lys Ala Ser Val Pro Gly Xaa
545 550 555 560

Xaa Xaa Xaa Xaa Gly Gly Asp Met Gly Gly Met Asp Phe
565 570

-47-

CLAIMS

1. A fusion protein comprising a stress protein fused to a protein against which an immune response is desired.
- 5 2. The fusion protein of Claim 1 wherein the stress protein is a heat shock protein and the protein is a human immunodeficiency viral protein.
3. The fusion protein of Claim 2 wherein the heat shock protein is hsp70 and the human immunodeficiency viral
10 protein is p24 protein.
4. A vaccine comprising all or a portion of a stress protein which induces an immune response in an individual to whom it is administered or all or a portion of a protein having an amino acid sequence
15 sufficiently homologous to the amino acid sequence of the stress protein to be capable of inducing an immune response in an individual to whom it is administered.
5. A vaccine of Claim 4 in which the stress protein is a
20 mycobacterial stress protein or a protein having an amino acid sequence sufficiently homologous to the amino acid sequence of the mycobacterial stress protein to induce an immune response in the individual to whom it is administered.
- 25 6. A vaccine for use in enhancing in an individual the immune response to a pathogen, comprising all or a portion of a stress protein of the pathogen against which the enhanced response is desired.

-48-

7. A vaccine of Claim 6 in which the stress protein is selected from the group consisting of: mycobacterial stress proteins, bacterial stress proteins, fungal stress proteins, viral stress proteins and parasitic stress proteins.
5
8. A composition comprising all or a portion of a selected stress protein, for use in producing or enhancing an immune response in an individual, wherein the stress protein is in sufficient quantity to elicit the desired immune response.
10
9. A composition comprising a stress protein for use in immunizing an individual against a subsequent infection by a pathogen, wherein the stress protein is in sufficient quantity to produce an immune response to the stress protein.
15
10. The composition of Claim 9 wherein the stress protein is a stress protein of the pathogen.
11. A composition comprising all or a portion of a stress protein or all or a portion of a protein having an amino acid sequence sufficiently homologous to the amino acid sequence of the stress protein for use in inducing in an individual immune tolerance against a protein, under conditions appropriate for induction of the desired tolerance.
20
12. A composition of Claim 11, wherein the protein is a protein associated with rheumatoid arthritis.
25
13. A vaccine for use in inducing an immune response in an individual comprising all or a portion of a stress protein or all or a portion of a protein having an

-49-

5 amino acid sequence sufficiently homologous to the amino acid sequence of the stress protein conjugated to a substance to which an immune response is desired or to a portion of the substance sufficient to induce an immune response in an individual.

10 14. A vaccine of Claim 13 in which the stress protein is a mycobacterial stress protein or a protein having an amino acid sequence sufficiently homologous to the amino acid sequence of the mycobacterial stress protein to induce an immune response in an individual to whom it is administered.

15 15. A vaccine of Claim 13 in which the substance against which an immune response is desired is selected from the group consisting of: proteins, peptides, oligosaccharides, lipids, carbohydrates, organic molecules and a combination thereof.

20 16. A vaccine for use in inducing an immune response in an individual comprising a recombinant fusion protein which includes all or a portion of a stress protein or all or a portion of a protein having an amino acid sequence sufficiently homologous to the amino acid sequence of the stress protein fused to a substance against which an immune response is desired or to a portion of the substance sufficient to induce an immune response in an individual.

30 17. A vaccine of Claim 16 in which the stress protein is a mycobacterial stress protein or a protein having an amino acid sequence sufficiently homologous to the amino acid sequence of the mycobacterial stress protein to induce an immune response in an individual to whom it is administered.

-50-

18. A vaccine of Claim 17 in which the protein is the HIV gag or pol protein.
19. A composition for use as an agent to induce immune tolerance, comprising a stress protein conjugated to
5 a substance to which an immune response is desired.
20. A vaccine for use in enhancing in an individual an immune response, comprising all or a portion of a stress protein conjugated to a substance to which an immune response is desired or to a portion of the
10 substance sufficient to enhance an immune response in the individual.
21. A vaccine of Claim 20 in which the stress protein is selected from the group consisting of: mycobacterial stress proteins, bacterial stress proteins, fungal
15 stress proteins, viral stress proteins and parasitic stress proteins.
22. A composition comprising a stress protein for use in producing or enhancing an immune response in an individual, wherein the stress protein is in
20 sufficient quantity to elicit the desired immune response, and the stress protein is conjugated to a substance against which an immune response is desired or to a portion of the substance sufficient to produce or enhance an immune response in the
25 individual.
23. A composition comprising a stress protein for use in immunizing an individual against a subsequent infection by a pathogen, wherein the stress protein is in sufficient quantity to produce an immune
30 response sufficient to protect the individual against

-51-

subsequent infection by the pathogen, and the stress protein is conjugated to a substance against which an immune response is desired or to a portion of the substance sufficient to produce an immune response in the individual.

5

24. A vaccine for use in inducing an immune response in an individual comprising all or a portion of a stress protein or all or a portion of a protein having an amino acid sequence sufficiently homologous to the amino acid sequence of the stress protein and a substance against which an immune response is desired or to a portion of the substance sufficient to induce an immune response in an individual.

10

25. A vaccine of Claim 24 in which the stress protein is a mycobacterial stress protein or a protein having an amino acid sequence sufficiently homologous to the amino acid sequence of the mycobacterial stress protein to induce an immune response in an individual to whom it is administered.

15

26. A vaccine of Claim 24 in which the substance against which an immune response is desired is selected from the group consisting of: proteins, peptides, oligosaccharides, lipids, carbohydrates, organic molecules and any combination thereof.

20

27. A composition for use as an agent to induce immune tolerance, comprising a stress protein and a substance to which an immune response is desired.

25

28. A vaccine for use in enhancing in an individual an immune response, comprising all or a portion of a stress protein and either a substance to which an

30

-52-

immune response is desired or a portion of the substance sufficient to enhance an immune response in the individual.

29. A vaccine of Claim 28 in which the stress protein is
5 selected from the group consisting of: mycobacterial stress proteins, bacterial stress proteins, fungal stress proteins, viral stress proteins and parasitic stress proteins.
30. A composition comprising a stress protein and a
10 substance against which an immune response is desired or a portion of the substance sufficient to produce or enhance an immune response in an individual for use in producing or enhancing an immune response in an individual, wherein the stress protein is in
15 sufficient quantity to elicit the desired immune response .
31. A composition comprising a stress protein and a
substance against which an immune response is desired
20 or to a portion of the substance sufficient to produce or enhance an immune response in the individual for use in immunizing an individual against a subsequent infection by a pathogen, wherein the stress protein is in sufficient quantity to produce an immune response sufficient to protect the
25 individual against subsequent infection by the pathogen.
32. A composition for use as an agent to induce an immune
response in an individual to whom it is administered,
30 comprising all or a portion of a stress protein or all or a portion of a protein having an amino acid sequence sufficiently homologous to the amino acid

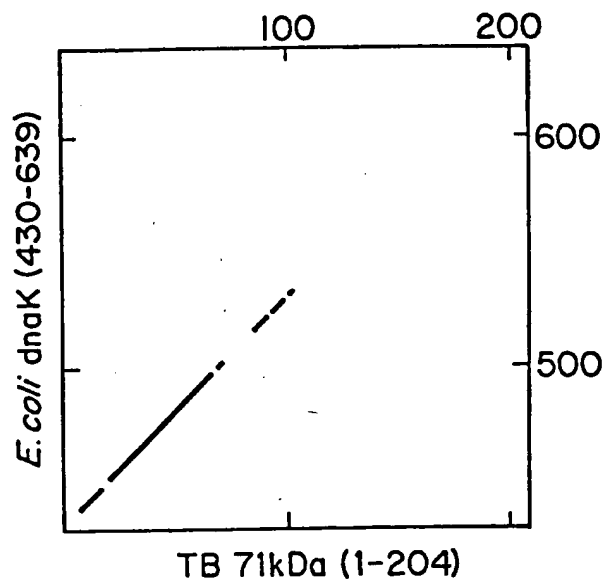
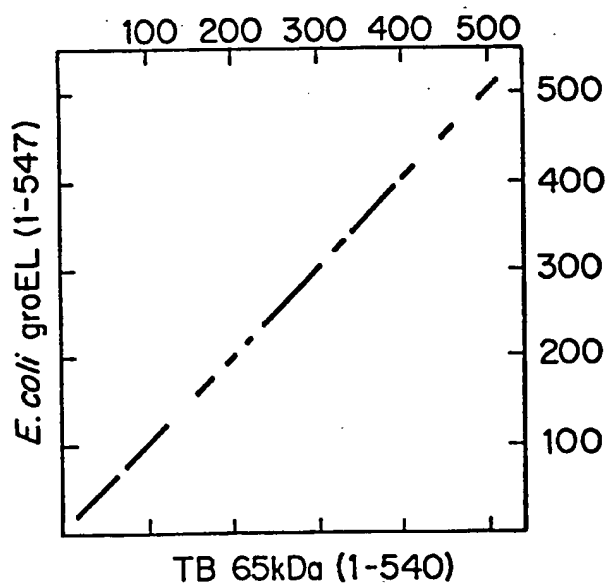
-53-

sequence of the stress protein to be capable of inducing an immune response in an individual to whom it is administered.

33. A composition for use as an agent to induce an immune response in an individual to whom it is administered, comprising all or a portion of a stress protein or all or a portion of a protein having an amino acid sequence sufficiently homologous to the amino acid sequence of the stress protein conjugated to a substance against which an immune response is desired or to a portion of the substance sufficient to induce an immune response in the individual.
34. A composition for use as an agent to induce an immune response in an individual to whom it is administered, comprising a recombinant fusion protein which includes a) all or a portion of a stress protein or all or a portion of a protein having an amino acid sequence sufficiently homologous to the amino acid sequence of the stress protein and b) a substance against which an immune response is desired or a portion of the substance sufficient to induce an immune response in the individual.
35. A composition for use as an agent to induce immune tolerance, comprising a stress protein.
36. A composition for use in treating an autoimmune disease, comprising all or a portion of a stress protein or all or a portion of a protein having an amino acid sequence sufficiently homologous to the amino acid sequence of the stress protein to induce immune tolerance in an individual to whom it is administered.

-54-

37. A composition of Claim 36 for treating rheumatoid arthritis.

*Fig. 1A**Fig. 1B*

1	10	20	30	40	50	60	70
HUM1	MLRLPTVFRQMRPVSRLAPHLTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGRTVIEEQSWGS						
GROEL	-----MA-----AKDVKFGNDARVKMLRGVNVNLADAVKVTGLGPKGRNVVLDKSFCA						
71	80	90	100	110	120	130	140
HUM1	PKVTKDGVTVAKSIDLKDKYKNIGAKLVQDVANNTNEEAGDGTATTATVLSIAKEGFEKISKGANPVEI						
GROEL	PTITKDGVSVAAREIEPEDKFKENMGAGQMVKEVASKANDAAGDGTATTATVLAQAIITEGLKAVAAGMNPMDL						
141	150	160	170	180	190	200	210
HUM1	RRGVNLAVDAVIAELKKQSKPVTTPPEIAQVATISANGDKEIGNIISDAMKKVGRKGVITVKDGKTLNDE						
GROEL	KRGIDKAVTAAVEELKALSVCSDSKAIAQVGTISANSDETVGKLIAEAMDKVKGEGVITVEDGTGLQDE						
211	220	230	240	250	260	270	280
HUM1	LEIEGKMFDRGYISPYFINTSKGQKCEFQDAYVLLSEKKISSIQSIVPALEIANAHKPLVIAEDVDG						
GROEL	LDVVEGMQFDRGYLSPYFINKPETGAVELESPFILLADKKISNIREMLPVLEAVAKAGKPLLIIEADVEG						
281	290	300	310	320	330	340	350
HUM1	EALSTLVNRLKVLQVAVKAPGFGDNRRKNQLKDMAIATGGAVFGEEGLTNLEDVQPHDLGKVGEVIV						
GROEL	EALATAVVNTIRGIVKVAAPGFGDRRKAMLQDIATLTGTVISEE-IGMELEKATLEDLGQAKRVVI						
351	360	370	380	390	400	410	420
HUM1	TKDDAMLLKGGKDAQIEKRIQEIIEQLDVTTSEYEKEKLNERLAKLSGDGVAVLKVGGTSDVEVNEKKDR						
GROEL	NKDTTIIIDGVGEEAAIQGRVAQIRQQIEEATSDYDREKLQERVAKLGGVAVIKVGAATEVEMKEKKAR						

FIGURE 2

```

421, 430, 440, 450, 460, 470, 480, 490,
HUMP1 VTDALNATRAAVEEGIVLGGGALLRCIPALDSLTPANEDQKIGIEIKRTLKIPAMTIKNAGVEGSLI
      : :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
GROEL VEDALHATRAAVEEGVAGGGVALIRVASKLADLRGQNEQNVVSSSL-RAMEAPLRQIVLNCGEEPSVV

491, 500, 510, 520, 530, 540, 550, 560,
HUMP1 VEKINQSSSEVGYDAMAGDFVNMVEKGIIDPTKVVRTALLDAAAGVASLLTTAEVVVVTEIPKEEKDPGMGA
      : :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
GROEL ANTVKGGDGNYGYNAAATEEYGNMIDMGILDPTKVRTSALQYAASVAGLMITTECMVTDLPKND-AADLGA

561, 570,
HUMP1 MGGMGG--GMGGGMF
      : :: :: :: :: ::
GROEL AGGMGGMGGMGGMM-

Total score = 4667, 5 breaks
276 identities out of 545 possible matches between residues
25 random runs
Alignment score = 65.34 SD Standard deviation = 18.94 Mean = 3429.48

```

FIGURE 2 (continued)

1	10	20	30	40	50	60	70
HUMP1	MLRLPTVFRQMRPVSRLAPHLTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGRTVITIEQSWGS						
ML65K	M-----AKTIAYDEEARGLERGLNSLADAVKVTDLGPKGRNVVLEKKWGA						
71	80	90	100	110	120	130	140
HUMP1	PKVTKDGVTVAKSIDLKDXYKNIGAKLVQDVANNTNEEAGDGTATTATVLSIAKEGFEKISKGANPVEI						
ML65K	PTITNDGVSIKAEIELEDPEYKIGAEELVKEVARKTDDVAGDGTATTATVLAQALVKEGLRNVAAAGANPLGL						
141	150	160	170	180	190	200	210
HUMP1	RRGVMLAVDAVIAELKKQSKPVTTPPEEIAQVATISANGDKEIGNIISDAMKKVGRKGVIITVKDGKTLNDE						
ML65K	KRGIEKAVDKVTETLLKDAKEVETKEQIAATAAISA-GDQSIGDLIAEAMDKVGNEGVITVEESNTFGLQ						
211	220	230	240	250	260	270	280
HUMP1	LEIIEGKMFDRGYISPYFINTSKGQKCEFDQDAYVLLSEKKISSIQSIVPALEIANAHKRKPLVIAEDVDG						
ML65K	LELTEGMRFDKGYISGYFVTDARQEAVLEEPYILLVSSKSVTVKDLLPLEKVIQAGKSLLIIAEDVEG						
281	290	300	310	320	330	340	350
HUMP1	EALSTLVNRLKVLQVAVKAPGFGDNRRKNQLKDMAIATGGAVFGEGLTLNLEDVQPHDLGKVGIV						
ML65K	EALSTLVVNKIRGTFKSVAVKAPGFGDRRKAMLODMAILTGAQVISEE-VGLTLENTDLSLLGKARKVVM						
351	360	370	380	390	400	410	420
HUMP1	TKDDAMLLKKGDKAQIEKRIQEIIEQLDVTTSEYEKEKLNERLAKLSDGVAVLKVGTSDEVEVNEKKDR						
ML65K	TKDETTIVEGAGDTDAIAGRVAQIRTEIENSDDSDYDREKLQERLAKLAGGVAVIKAGAAATEVELKERKHR						

FIGURE 3

5/10

421	430	440	450	460	470	480	490
HUMP1	VTDALNATRAAVEEGIVLGGGCALLRCIPALDSLT	TPANEDQKIGIEIIKRTLKIPAMTI	AKNAGVEGSLI				
ML65K	IEDAVRNAKAAVEEGIVAGGGVTLQAA	PALDKLKLGTDEAT-GANIVKVALEAPL	KQIAFNSGMEPGVV				
491	500	510	520	530	540	550	560
HUMP1	VEKIMQSSSEVGVDAMAGDFVNMVEKGIIDPTKVV	TALLDAAGVASLLTTAEVVVTEIPKEKDPGMGA					
ML65K	AEKVRNLSVGHGLNAATGEYEDLLKAGVADPVK	TRSALQNAASIAGLFTT-EAVVADKPEKTAAPASDP					
561	570						
HUMP1	MGGMGGMGGGMF						
ML65K	TGGMGG-MD---F						

Total score = 4552, 7 breaks
 255 identities out of 540 possible matches between residues
 25 random runs
 Alignment score = 47.73 SD Standard deviation = 23.86 Mean = 3413.16

FIGURE 3 (continued)

1	10	20	30	40	50	60	70
HUMP1	MLRLPTVFRQMRPVSRLAPHLTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGRTVIIIEQSWGS						
TB65K	M-----AKTIAYDEEARRGLERGLNALADAVKVTGLGPKGRNVVLEKKWGA						
71	80	90	100	110	120	130	140
HUMP1	PKVTKDGVTVAKSIDLKDKNIGAKLVQDVANNTNEEAGDGTATTATVLARSIAKEGFEKISKGANPVEI						
TB65K	PTITNDGVSIKAKEIELEDPYEKIGAEVLVKEVAKKTDDVAGDGTATTATVLAQALRKEGLRNVAAGANPLGL						
141	150	160	170	180	190	200	210
HUMP1	RRGVMLAVDAVIAELKKQSKPVTTPPEEIAQVATISANGDKKEIGNIISDAMKKVGRKGVTITVKDGKTLNDE						
TB65K	KRGIEKAVEKVTTETLLKGAKEVETKEQIAATAAISA-GDQSIGDLIAEAMDKVGNEGVITVEESNTFGLQ						
211	220	230	240	250	260	270	280
HUMP1	LEIIEGKMFDRGYISPYFINTSKGQKCEFDAYVLLSEKKISSIQSIVPALEIANAHRKPLVIIAEDVDG						
TB65K	LELTEGMRFDKGYISGYFVTDPERQEAHVLEDPYILLVSSKSVSTVKDILLPLEKVIKAGKPLIIAEDVEG						
281	290	300	310	320	330	340	350
HUMP1	EALSTLVNRLKVLQVAVKAPGFGDNRRKNQKDMAIATGGAVFGEGLTLNLEDVQPHDLGKVGIVIV						
TB65K	EALSTLVNKRIRGTFKSVAVKAPGFGDRRKAMLQDMAILTGGQVISEE-VGLTLENADLSLLGKARKVVV						
351	360	370	380	390	400	410	420
HUMP1	TKDDAMLLKGKDKAQIEKRIQEIIEQLDVTTSEYEKEKLNERLAKLSDGVAVLVKVGSTSDVEVNEKKDR						
TB65K	TKDETTIVEGAGDTDAIAGRVAQIRQEIENSDDYDREKLQERLAKLAGGVAVIKAGAAATEVELKERKHR						

FIGURE 4

7/10

```

421      430      440      450      460      470      480      490
HUMP1    VTDALNATRAAVEEGIVLGGGCALLRCIPALDSLTPANEDQKIGIEIIKRTLKIPAMTIAKNAGVEGSLI
TB65K    IEDAVRNAKAAVEEGIVAGGGVTLQAAFTLDELK--LEGDEATGANIVKVALEAPLKQIAFNSGLEPGVV

491      500      510      520      530      540      550      560
HUMP1    VEKIMQSSSEVGYDAMAGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLTTAEVVVTEIPKEEKDPGMGA
TB65K    AEKVRNLPAGHGLNAQTGVYEDLLAAGVADPVKVTRSALQNAASIAGLFLTTEAVVADKPEKEKASVPG-

561      570
HUMP1    MGGMGGMGGGMF
TB65K    ----GGDMGGMDF

Total score = 4560, 5 breaks
257 identities out of 540 possible matches between residues
25 random runs
Alignment score = 49.36 SD Standard deviation = 23.23 Mean = 3413.16

```

FIGURE 4 (continued)

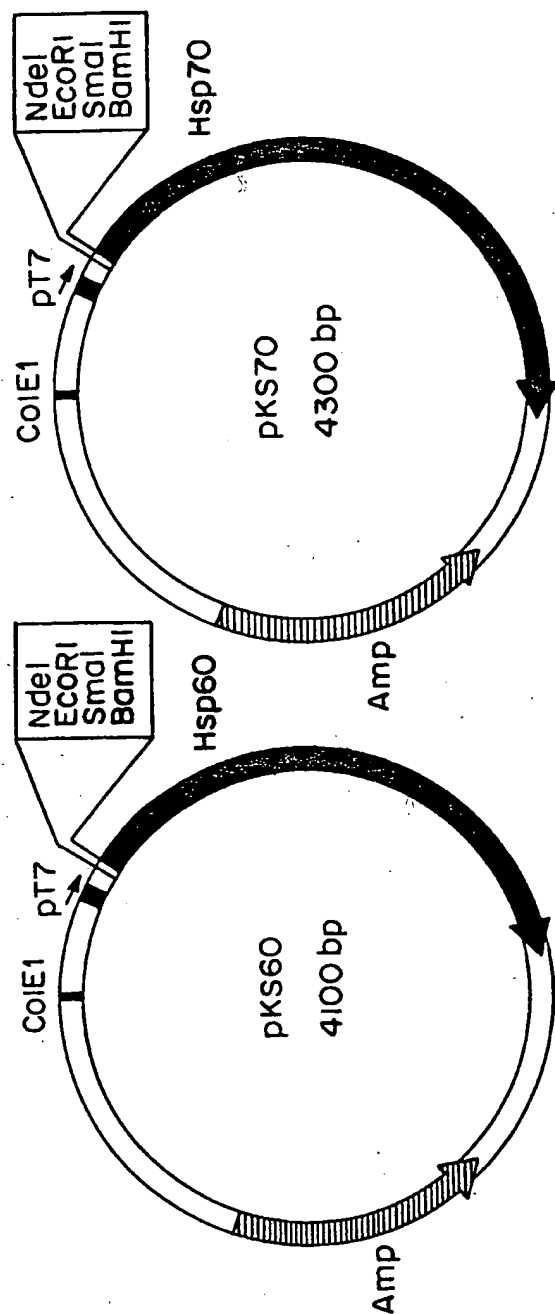


FIG. 5

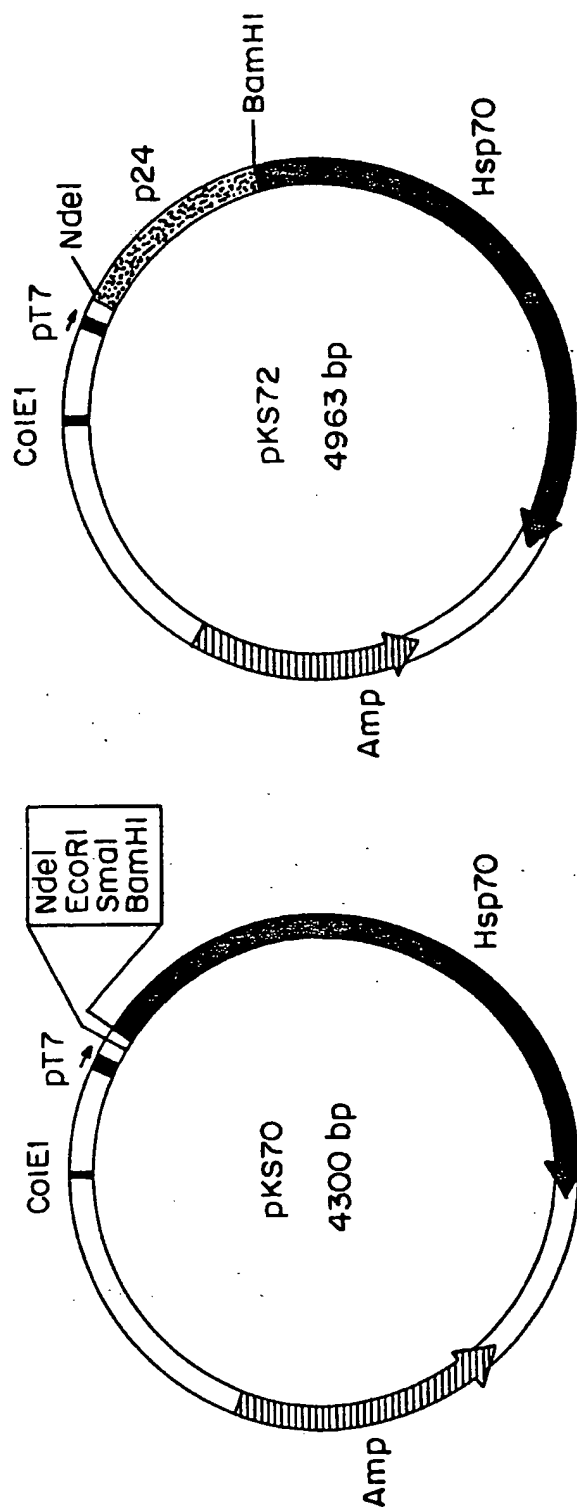


FIG. 6

10/10

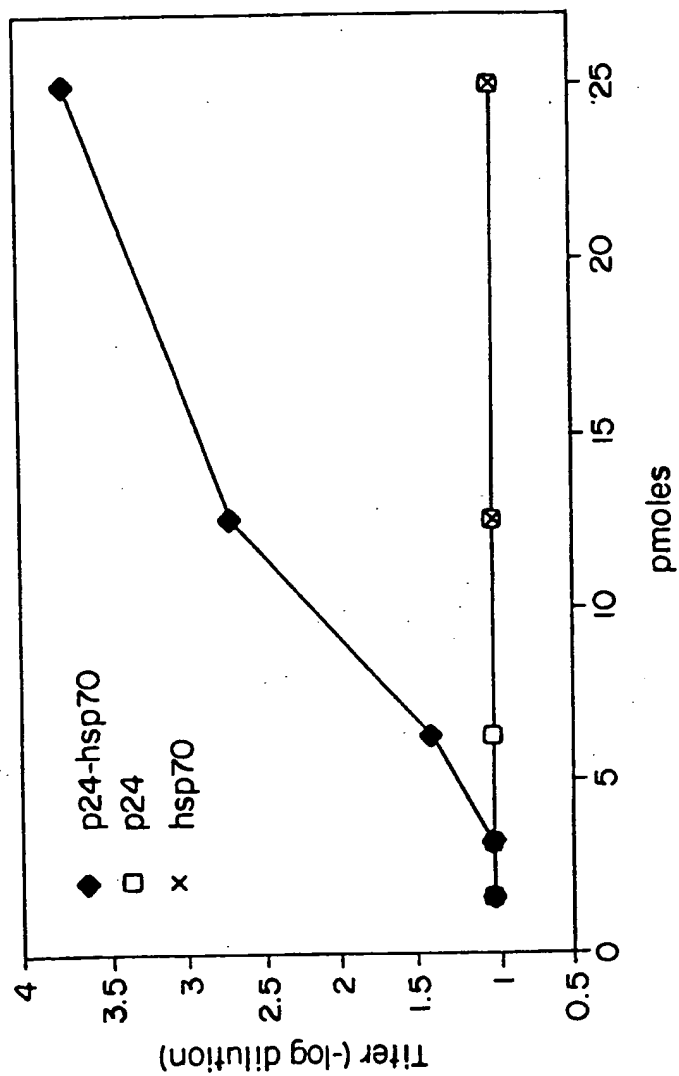


FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/06362

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/62 C07K15/04 A61K39/295 A61K39/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,89 12455 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 28 December 1989 see the whole document --- -/--	4-12, 32, 35-37

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

15 September 1994

Date of mailing of the international search report

13 -10- 1994

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Fuhr, C

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/06362

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>EUROPEAN JOURNAL OF IMMUNOLOGY vol. 22, no. 6, June 1992, WEINHEIM, DE pages 1365 - 1372 C. BARRIOS ET AL. 'Mycobacterial heat-shock proteins as carrier molecules. II: The use of the 70-kDa mycobacterial heat-shock protein as carrier for conjugated vaccines can circumvent the need for adjuvants and Bacillus Calmette Guérin priming' cited in the application see page 1366, left column, paragraph 4 -paragraph 5 see page 1366, right column, paragraph 3 see page 1368, left column, paragraph 1 - page 1370, right column, paragraph 1 see page 1371, left column, last paragraph - right column, paragraph 1 ----</p>	13-17, 19-31,33
P,X	<p>WO,A,93 17712 (BIOCINE SCLAVO SPA) 16 September 1993 see claims; examples ----</p>	13-17, 19-31,33
P,X	<p>WO,A,94 03208 (YEDA RESEARCH AND DEVELOPMENT COMPANY LTD.) 17 February 1994 see page 8, paragraph 3; claims ----</p>	13-17, 19-31,33
A	<p>WO,A,90 15873 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 27 December 1990 see the whole document -----</p>	1-3, 13-31, 33,34

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 94/06362

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8912455	28-12-89	EP-A- 0419569	03-04-91
WO-A-9317712	16-09-93	NONE	
WO-A-9403208	17-02-94	AU-B- 4790093	03-03-94
WO-A-9015873	27-12-90	AU-A- 5848090	08-01-91
		CA-A- 2063414	20-12-90
		EP-A- 0478664	08-04-92
		JP-T- 4506297	05-11-92